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14. **ABSTRACT**
One of the principal objectives of our laboratory is to understand the mechanisms by which hormones and growth factors regulate normal mammary gland development, and how these same regulatory pathways become altered in breast cancer. Therefore, specific interest has been placed upon studying the mechanisms by which the lactogenic hormones regulate milk protein gene expression. CAAT/enhancer binding protein β (C/EBPβ), Yin Yang-1 (YY-1), signal transducers and activators or transcription 5 (STAT5), and the glucocorticoid receptor (GR) have been shown to mediate the hormonal and developmental regulation of a β-casein gene. However, the mechanism by which these transcription factors as well as coactivator proteins coordinately function to promote normal mammary gland development and breast cancer remains undefined. In this study we tested the dynamic of assembly and disassembly of Stat5, GR, C/EBPb and YY-1 at the hormonally activated b-casein promoter as well as at the recently identified mouse b-casein enhancer located –6kb upstream of the transcription start site. Using ChIP analysis we examined the recruitment of coactivator p300 and determined chromatin acetylation and deacetylation status upon hormone treatments. Finally, we established the time course of pol II and phospho-pol II accumulation at the promoter and enhancer sites. Collectively, these data suggest a model for the assembly of multi-protein complex at the b-casein regulatory regions that helps to understand how the signaling pathways regulated by lactogenic hormones and local growth factors are integrated in the nucleus.

15. SUBJECT TERMS
ß-casein, C/EBPß, YY-1, STAT5, GR, mammary gland development, ChIP assay

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Introduction

We have postulated that lactogenic hormones and local growth factors stimulate β-casein gene transcription through a specific and ordered assembly of transcription factors and co-modulatory proteins. Previous transient transfection experiments, as well as studies in mouse models, have revealed the importance of Stat5, C/EBPß and GR in the regulation of milk protein gene expression. However, their separate and combinatorial roles in the recruitment of additional co-regulatory factors necessary for the effective transcriptional regulation have not yet been defined. Which co-regulatory factors are present in this multiprotein complex has also not been determined. Transcriptional activation/repression is generally correlated with histone acetylation/deacetylation. Orchestration of all the events required for transcriptional activation (recruitment of transcription factors, chromatin modification and remodeling, an assembly of the preinitiation complex) is promoter specific. Our focus is on two distinct regions of the milk protein gene β-casein including a promoter region, which is near the transcription start site, and an enhancer region, which is 6 kb upstream of the transcription start site. We employed ChIP assays to examine transcription factor interactions at the ß-casein proximal promoter and upstream distal enhancer in the native chromatin of mammary epithelial HC11 cells following treatment with prolactin alone, glucocorticoids alone, or both hormones.

Body

To understand the role of Stat5, GR, C/EBPß and co-modulatory factors in hormonally-regulated chromatin remodeling at the ß-casein promoter and enhancer.

1a. RNA analysis

These experiments were performed in mammary epithelial HC11 cells, which are known to express β-casein in the presence of lactogenic hormones (1). Total RNA was isolated from untreated cells and cells treated with prolactin (Prl) alone, hydrocortisone

(HC) alone, or both hormones for different periods of time (Figure1A). After DNAse I treatment, total RNA was reverse transcribed and amplified by PCR using exon VII primers specific for the mouse β-casein gene. Treatment with HC alone, which activates the glucocorticoid receptor (GR), produced no increase in casein mRNA levels. A small increase in β-casein RNA accumulation at 24 hrs was detected in cells treated with Prl alone, which activates Stat5. However, a large increase (>500-fold) in β-casein mRNA occurred when cells were treated with both hormones as measured by quantitative RT-PCR (Figure1B). These experiments were performed in parallel to ChIP assays and served as a basic control for β-casein gene expression.

1b. The involvement of histone acetylase and histone deacetylase activity in activation of β-casein transcription.

To explore the possibility that β-casein expression is regulated by changes in chromatin structure, we examined histone acetylation and histone deacetylation activity using ChIP assay with antibodies against acetylated histone H3 and α-HDAC1 (Figure 2).

We have been able to demonstrate rapid acetylation (in 15 minutes) at both the promoter and enhancer after stimulating cells with prolactin and hydrocortisone. In cells stimulated with prolactin alone, we did not see any changes in histone H3 acetylation compared to non-treated cells. Surprisingly, in cells treated with hydrocortisone alone we observed a rapid 12-to15-fold hyper-acetylation of histone H3 at both the promoter and enhancer regions after 15 minutes of treatment as compared to non-stimulated cells. The observed hyperacetylation at both regions of the β-casein gene confirms the hypothesis that GR may have a multiple functions: it may initiate the chromatin remodeling required for transcription initiation, and at the same time it may play a bridging role in β-casein activation through interactions with Stat5 and C/EBPβ as well as binding to different co-activators, co-modulators and/or co-repressors.

Interestingly, we were also able to detect an increased level of histone deacetylation 15 minutes after stimulation of cells with prolactin alone or in combination with hydrocortisone (Figure 2). However, in cells treated with hydrocortisone only, we did not observe any changes in HDAC activity after 15 min of treatment. These results suggest that Stat5, but not GR, is responsible for the transient increase observed in

HDAC1 and deacetylase activity associated with the induction of β-casein gene expression. It has been recently demonstrated that Stat5-induced transcription on some promoters may involve recruitment of HDAC1 and deacetylation of C/EBPβ (2,3).

A summary of the histone acetylation and deacetylation status of chromatin after hormone stimulation at different times (0 to 24hrs) assayed using real-time qPCR is shown in Figure 3.

1c. Recruitment of transcription factors Stat5, GR, C/EBPb and YY-1 in chromatin remodeling.

In order to better understand how lactogenic hormones regulate milk protein gene expression at the β-casein promoter and enhancer, we used antibodies to different transcription factors in modified ChIP assays and employed real time qPCR for quantitative analysis following treatment with Prl alone, HC alone, or both hormones (Figures 4 and 5).

By performing ChIP/Western experiments we were able to demonstrate the recruitment of Stat5, GR and C/EBPß after stimulation of HC11 cells with hormones (Figure 4A,B). Rapid accumulation (in 15 min) of all three transcription factors was detected at the proximal promoter after stimulation of cells with hydrocortisone and prolactin (Figure 4C).

ChIP assays using a GR antibody in cells treated with hydrocortisone alone or in cells treated with both hormones, showed a transient increase in chromatin association (2-fold) at the proximal promoter region between 5 to 30 minutes following hormone addition, followed by cyclic changes. Slightly different dynamics of GR accumulation were observed at the distal enhancer. In cells treated with prolactin alone, we did not detect any changes in GR binding compare to untreated cells at both regions, as expected.

The time course of assembly and disassembly of Stat5 at the β-casein promoter and enhancer in cells treated with hydrocortisone, or prolactin, or the combination of both hormones is showed on Figure 5. Treatment with prolactin alone resulted in elevated (2.5-fold) accumulation of Stat5 at 15min that was diminished to basal level by 24 hours. However, there was a much greater accumulation of Stat5 at the promoter (9.5-fold) by 15 minutes in cells treated with both prolactin and hydrocortisone. No Stat5 accumulation was detected in cells stimulated with hydrocortisone alone, as expected.

ChIP assays using an anti-YY-1 antibody in HC11 cells following prolactin treatment showed a rapid disassociation of YY-1 from the □-casein promoter (Figure 9A). Maximal dissociation of YY-1 in cells treated with both hormones was observed by 15 to 30 minutes. No changes in the dynamics of YY-1 accumulation were observed in cells treated with hydrocortisone alone. These results suggest that Stat5 induced by prolactin is responsible for the disassociation of YY-1 from the □-casein promoter. As depicted in Figure 6B, in HC11 cells treated with prolactin, Stat5 and YY-1 display a reciprocal relationship in their association with the ß-casein promoter: when Stat5 binding increases, YY-1 decreases. These data are consistent with earlier studies from our laboratory and that of Bernd Groner's, which suggested that YY-1 represses □-casein gene expression in the absence of prolactin, and lactogenic hormones act by relieving this repression.

1d. Recruitment of p300 and RNA Polymerase II.

ChIP assays using antibodies to p300 (Figure 10) in cells treated with hydrocortisone alone showed the recruitment of this co-activator/co-modifier at both the proximal promoter and distal enhancer regions with a maximal association at 5minutes. However, in cells treated with prolactin maximal association was shifted to 15 minutes. Interestingly, in cells treated with both hormones a cumulatively increased association with both promoter and enhancer was observed. These results suggest that co-activator/co-modifier p300 is involved into the assembly of the multi-protein complex on activated □-casein through protein:protein interactions with GR and Stat5.

Time course of the recruitment of polymerase-II and phospho-polimeraseII at □-casein promoter and enhancer in cells treated with hydrocortisone and prolactin is showed on Figure 11. We observed an increased level of RNA polymerase II association with activated □-casein in 5minutes after stimulation with hormones with no significant changes in a time course up to 24 hrs. The dynamic of phospho-polimeraseII accumulation however, was different with a maximum at 1hr on promoter and even later on enhancer.

We summarized the results from the study and attempted to integrate them into a working model for the assembly of multi-protein complex at the □-casein regulatory

regions (Figure 12), which helps to understand how the signaling pathways regulated by lactogenic hormones and local growth factors are integrated in the nucleus.

Key Research Accomplishments

- ChIP in HC11 cells revealed the kinetics of transcriptional activation at β-casein proximal promoter and distal enhancer.
- Rapid histone acetylation was detected at both the promoter and enhancer regions of β-casein gene followed stimulation of cells with hydrocortisone alone or in combination with prolactin (but not with prolactin alone).
- GR is involved in initial chromatin remodeling events, including H3 acetylation.
- Histone deacetylation activity in a first 15 minutes after treatment cells with prolactin alone or in combination with hydrocortisone (but not with hydrocortisone alone) was detected at both the promoter and enhancer regions of β-casein.
- HDAC1 is possibly involved in deacetylation/activation events through the recruitment of transcription factors like C/EBPβ
- Prl induced recruitment of STAT5 on the promoter and enhancer is required for β-casein expression. Recruitment is augmented by GR and acetylation events.
- YY-1 represses β-casein gene expression in the absence of lactogenic hormones, but the repression is relieved followed prolactin treatment.
- Co-activator/co-modifier p300 is involved in transcription factors assembly and chromatin remodeling events occurring at the hormonally-activated β-casein gene through the protein:protein interactions: an increased level of p300 binding to the promoter and enhancer was determined in cells treated with prolactin alone, hydrocortisone alone and with both hormones as compared to non-treated cells.
- The accumulation of RNA polymerase-II at the β-casein promoter and enhancer was detected after 5 minutes of stimulation with hormones. However, the accumulation of p-Pol II occurred only at a later time (after 30 minutes) had its peak at 1hour and then declined.

Conclusions

Activation of signal transduction pathways by lactogenic hormones together with local growth factors, cell-cell and cell-substratium interactions result in specific transcription complex formation that changes chromatin structure and promote the expression of milk protein gene β-casein. In this study we elucidated the dynamics of different events resulting in transcriptional activation of β-casein gene expression through the evaluation of its regulatory regions (proximal promoter and distal enhancer) by performing ChIP assays in HC11 cells followed by stimulation with hydrocortisone alone, prolactin alone, or both hormones. We examined the dynamics of assembly/disassembly of basic transcription factors such as Stat5, GR, C/EBPß and YY-1, as well as the recruitment of co-activator/co-modifier p300. We also determined histone acetylation /deacetylation events and established the time course of RNA pol II and p-pol II accumulation at the hormonally activated β-casein gene promoter and enhancer. These results should help us understand better the mechanisms by which hormones and growth factors regulate normal mammary gland development and how these same regulatory pathways become altered in breast cancer. The ChIP assay developed in this study and optimized for HC11 cells has already been successfully extended for lactating mammary gland tissue.

Reportable Outcomes

Presentaions/Abstracts:

Xian, W., Kabotyanski E.B., and Rosen, J.M. "Hormonal Regulation of Mammary Gland Development and Breast Cancer". The Fourth Era of Hope Meeting, Philadelphia, Pennsylvania, June 8-11, 2005.

Manuscripts:

Kabotyanski E.B., **Xian, W.**, and Rosen, J.M. Specific and ordered assembly of C/EBP, STAT5, GR and comodulatory factors to the proximal promoter and distal enhancer of β-casein gene upon hormone stimulation. *Manuscript in preparation*.

Funding applied for, based on work supported by this award:

We have used the work presented in this report to successfully apply for a competitive renewal of NIH grant CA16303.

Appendix: Current contact information

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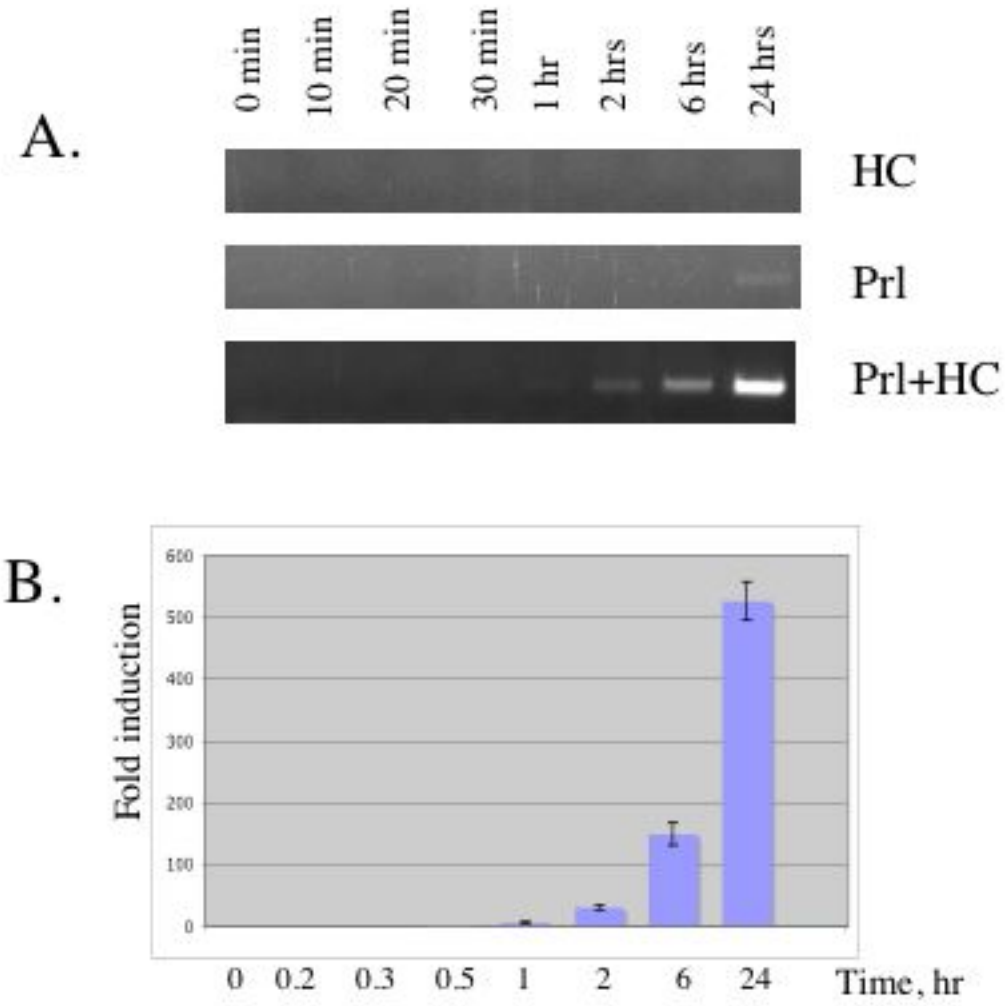

A.
0 min
10 min
20 min
30 min
1 hr
2 hrs
6 hrs
24 hrs
HC
Prl
Prl+HC
B.
Fold induction
600
500
400
300
200
100
0
0
0.2
0.3
0.5
1
2
6
24
Time, hr

Figure 1

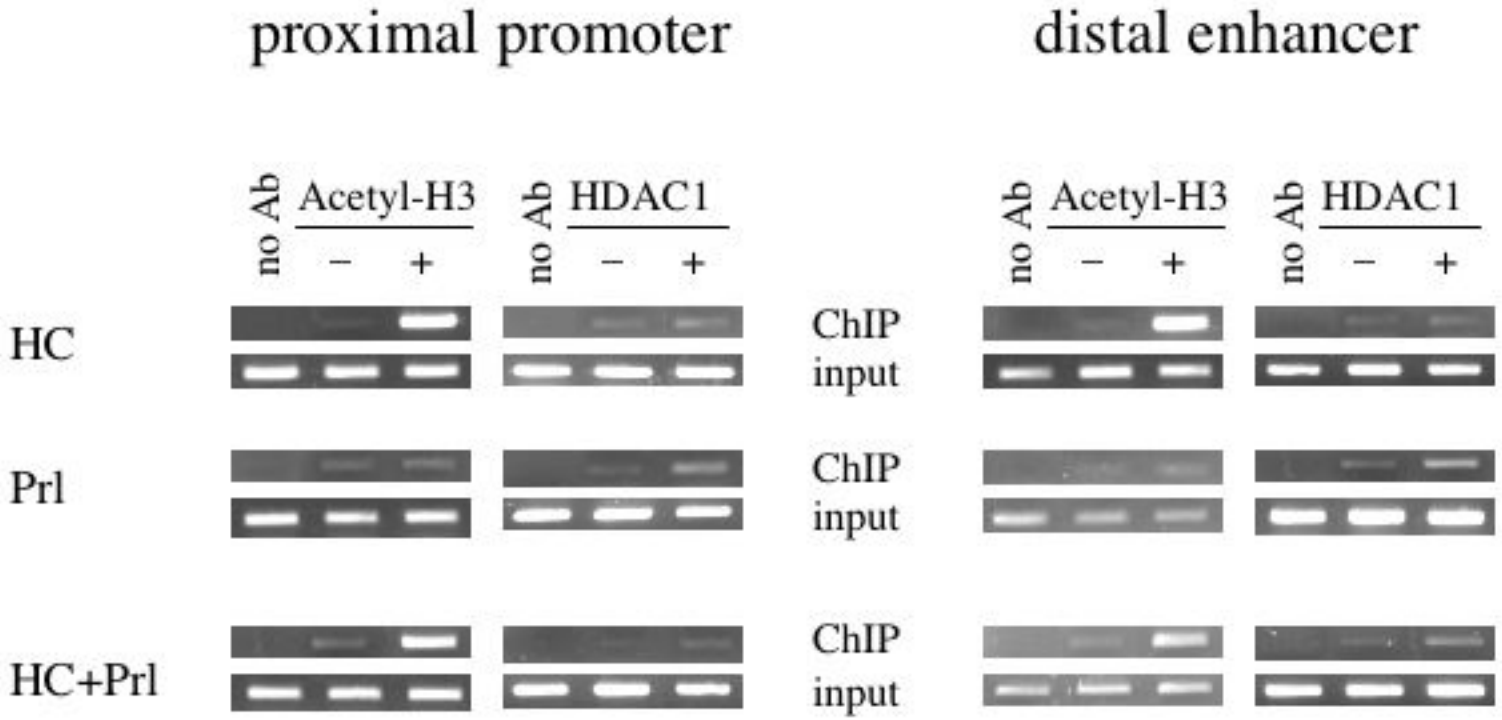

proximal promoter
distal enhancer
no Ab
Acetyl-H3
HDAC1
−
+
HC
Prl
HC+Prl
ChIP
input

Figure 2

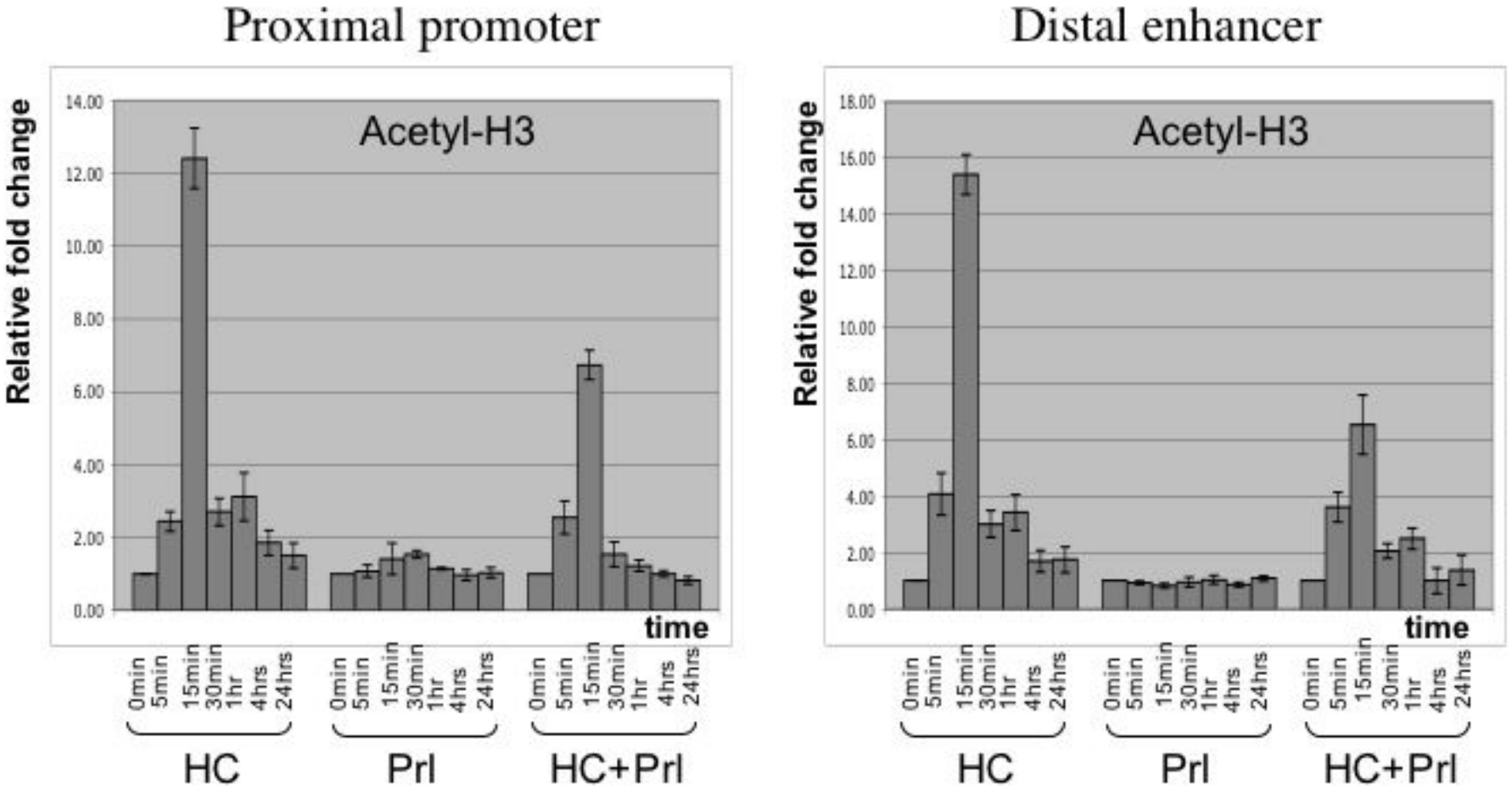

Proximal promoter
Distal enhancer
Acetyl-H3
Acetyl-H3
Relative fold change
Relative fold change
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
16.00
time
time
0min
5min
15min
30min
1hr
4hrs
24hrs
HC
Prl
HC+Prl

Figure 3

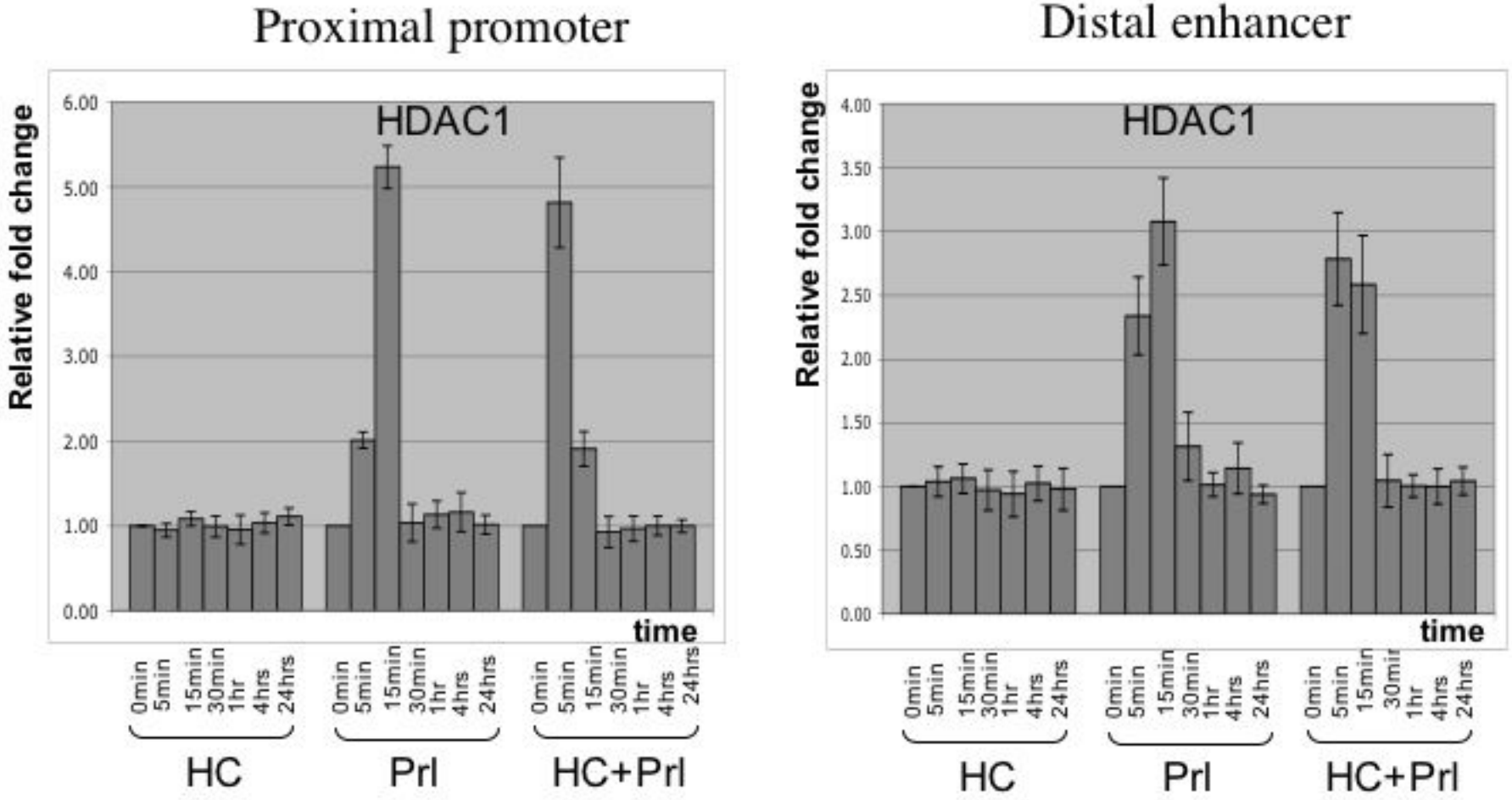

Proximal promoter
Distal enhancer
HDAC1
HDAC1
Relative fold change
Relative fold change
6.00
5.00
4.00
3.00
2.00
1.00
0.00
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
time
time
0min
5min
15min
30min
1hr
4hrs
24hrs
HC
Prl
HC+Prl

Figure 4

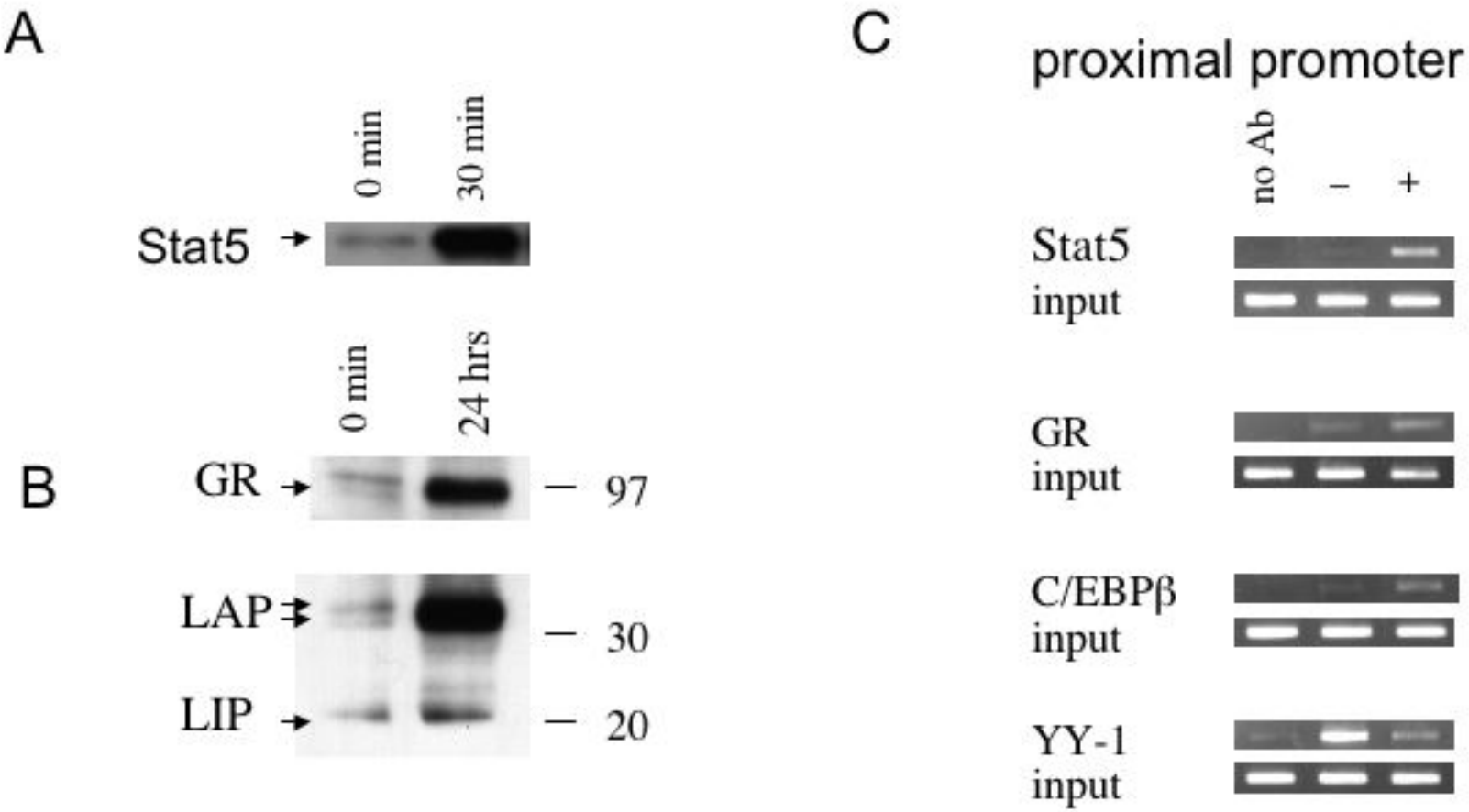

A
0 min
30 min
Stat5
B
0 min
24 hrs
GR
97
LAP
30
LIP
20
C
proximal promoter
no Ab
–
+
Stat5
input
GR
input
C/EBPβ
input
YY-1
input

Figure 5

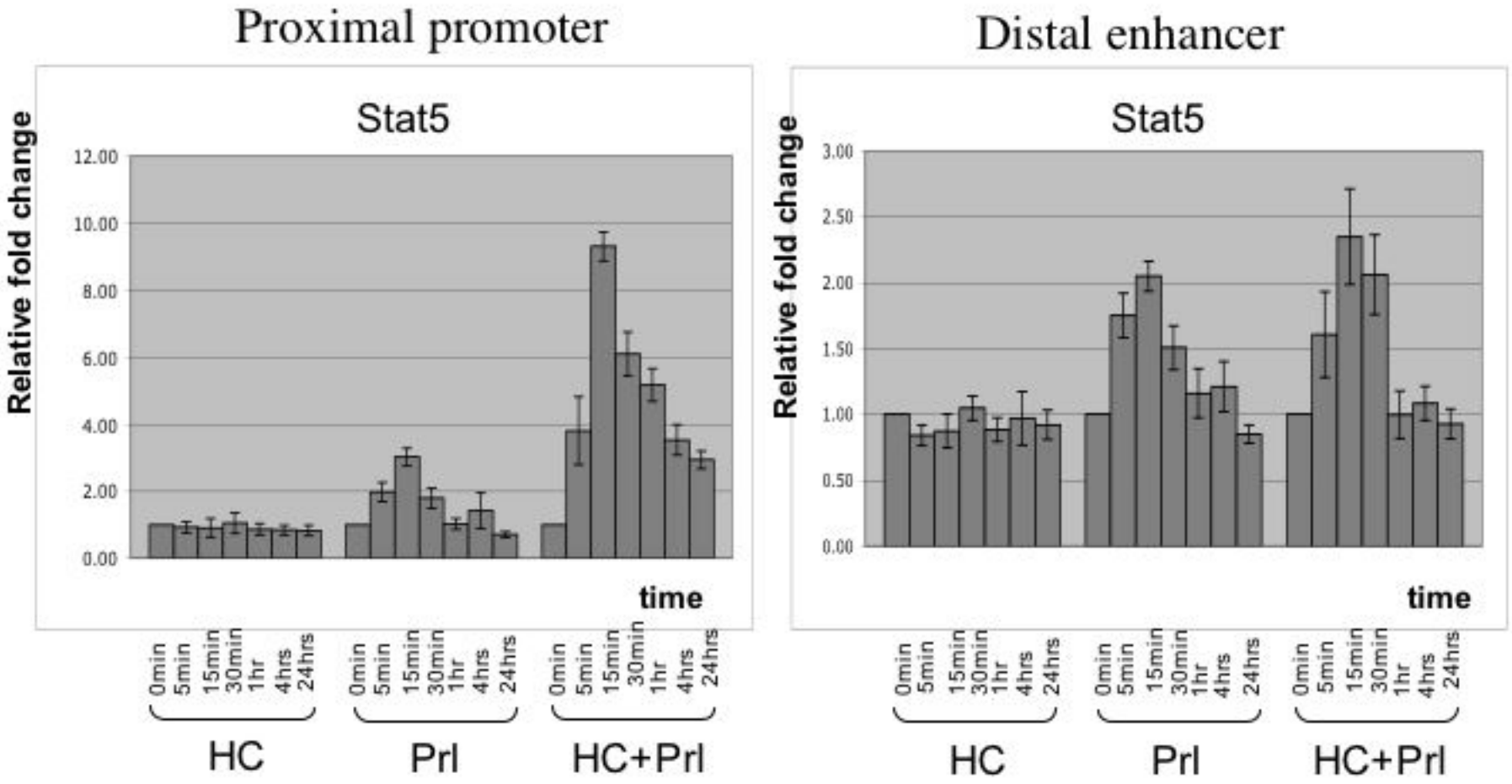

Proximal promoter
Stat5
Relative fold change
12.00
10.00
8.00
6.00
4.00
2.00
0.00
time
0min 5min 15min 30min 1hr 4hrs 24hrs
HC
Prl
HC+Prl
Distal enhancer
Stat5
Relative fold change
3.00
2.50
2.00
1.50
1.00
0.50
0.00
time
0min 5min 15min 30min 1hr 4hrs 24hrs
HC
Prl
HC+Prl

Figure 6

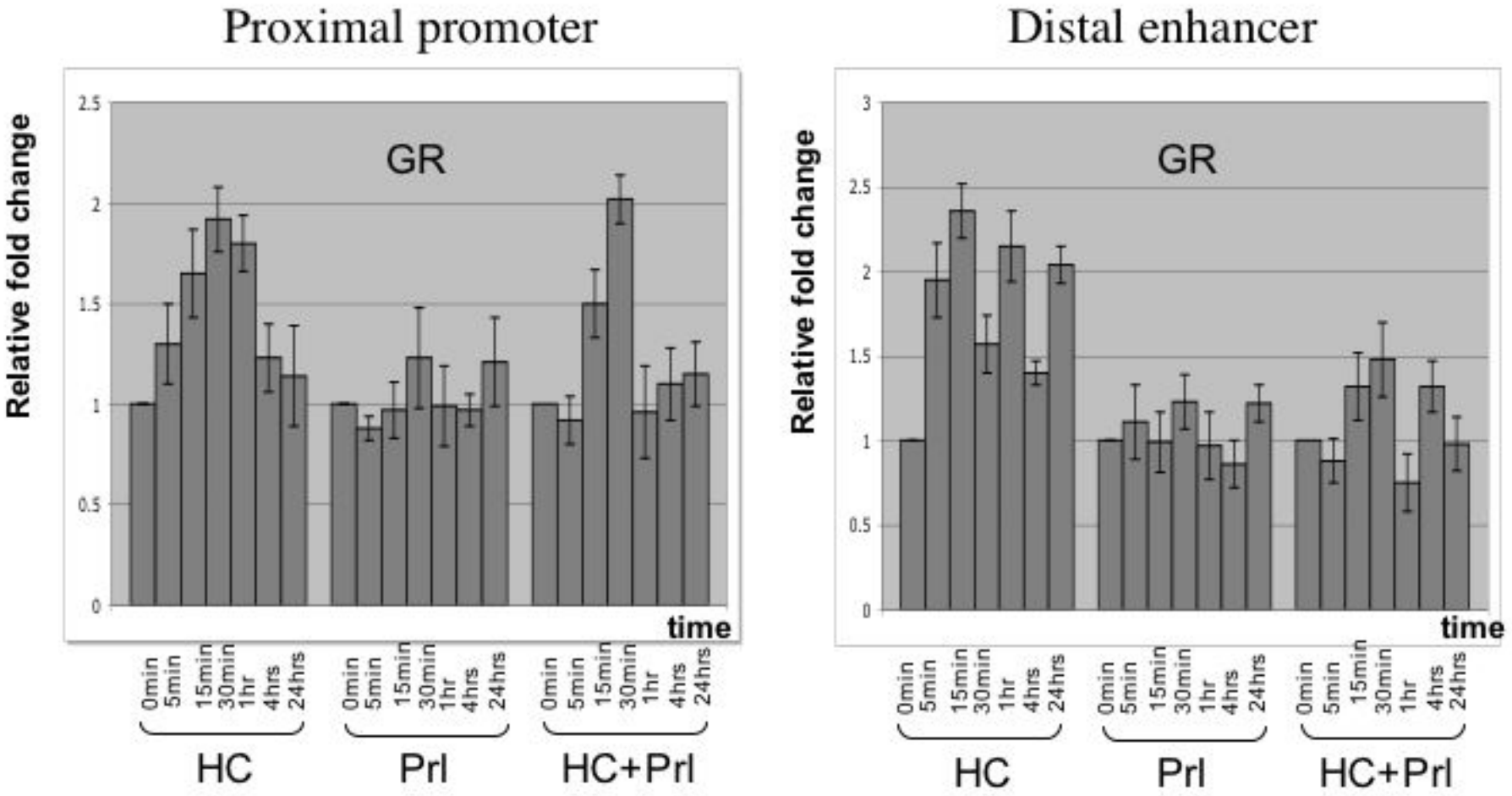

Proximal promoter
Distal enhancer
GR
GR
Relative fold change
Relative fold change
time
time
0min
5min
15min
30min
1hr
4hrs
24hrs
HC
Prl
HC+Prl

Figure 7

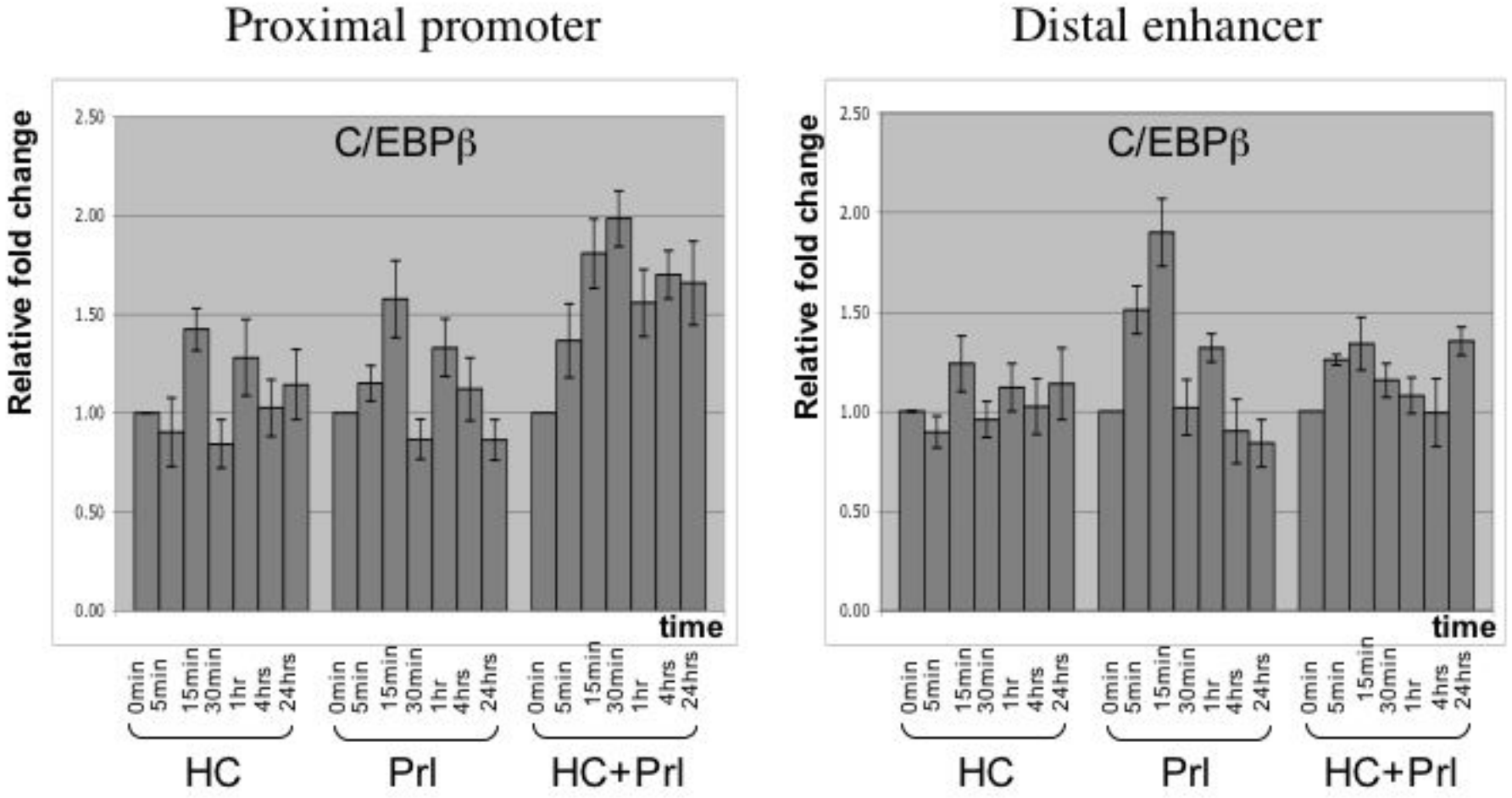
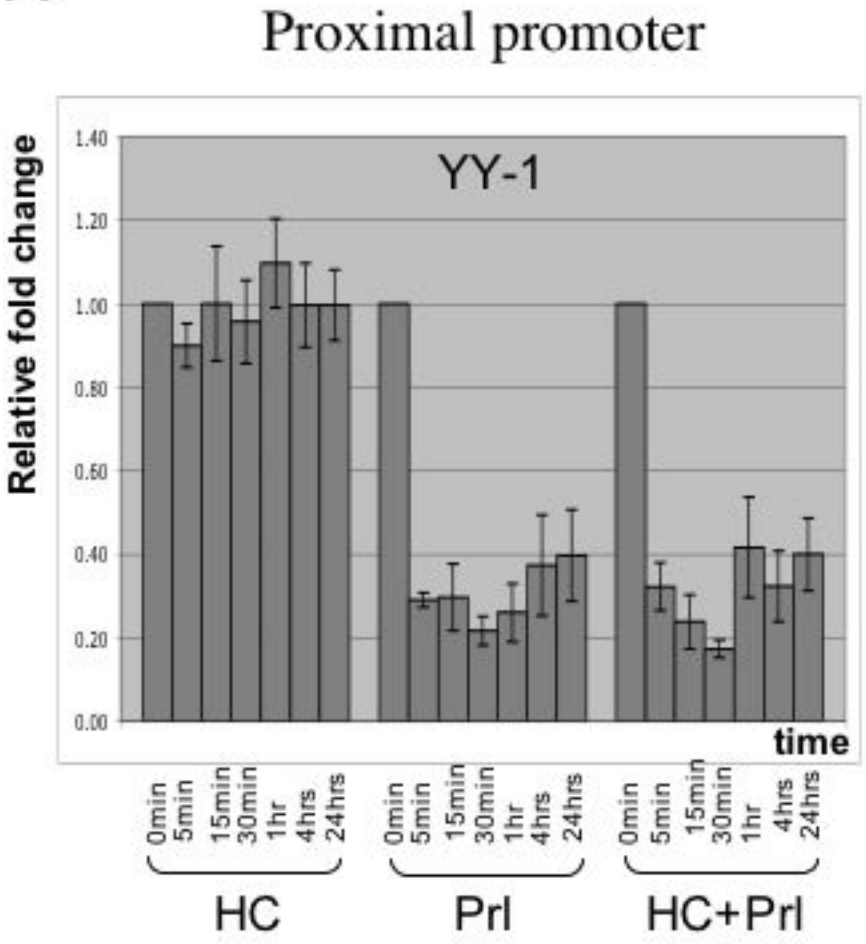

Proximal promoter
Distal enhancer
C/EBPβ
C/EBPβ
Relative fold change
Relative fold change
2.50
2.00
1.50
1.00
0.50
0.00
time
time
0min
5min
15min
30min
1hr
4hrs
24hrs
HC
Prl
HC+Prl

Figure 8

A


Proximal promoter
YY-1
Relative fold change
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
time
0min
5min
15min
30min
1hr
4hrs
24hrs
HC
Prl
HC+Prl

B

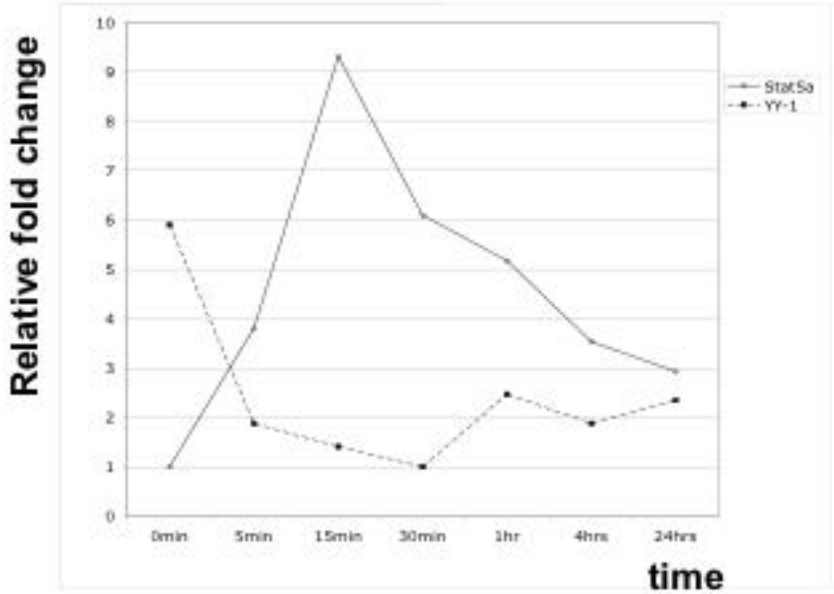

Relative fold change
10
9
8
7
6
5
4
3
2
1
0
0min
5min
15min
30min
1hr
4hrs
24hrs
time
Stat5a
YY-1

Figure 9

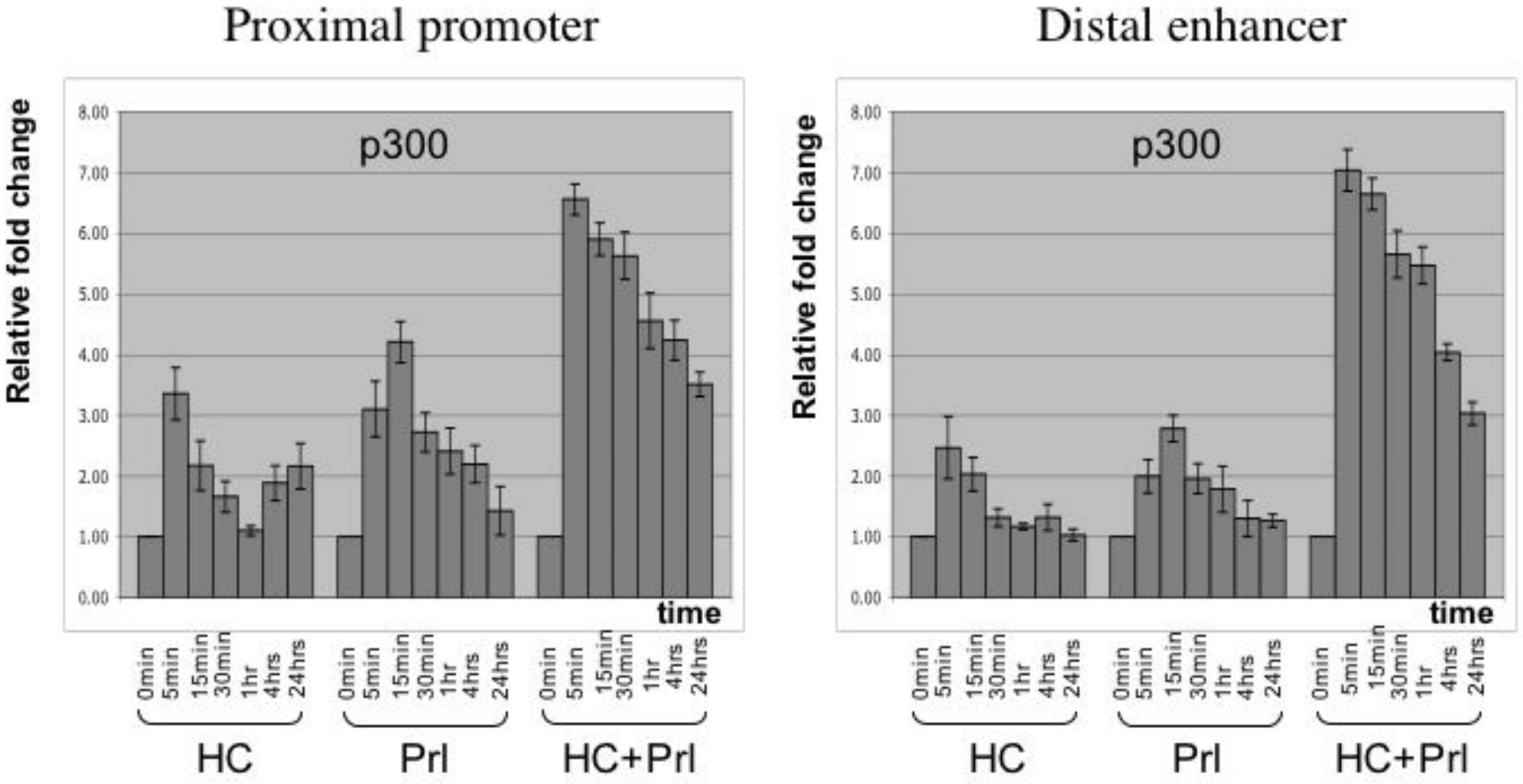

Proximal promoter
Distal enhancer
p300
p300
Relative fold change
Relative fold change
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
time
time
0min
5min
15min
30min
1hr
4hrs
24hrs
HC
Prl
HC+Prl

Figure 10

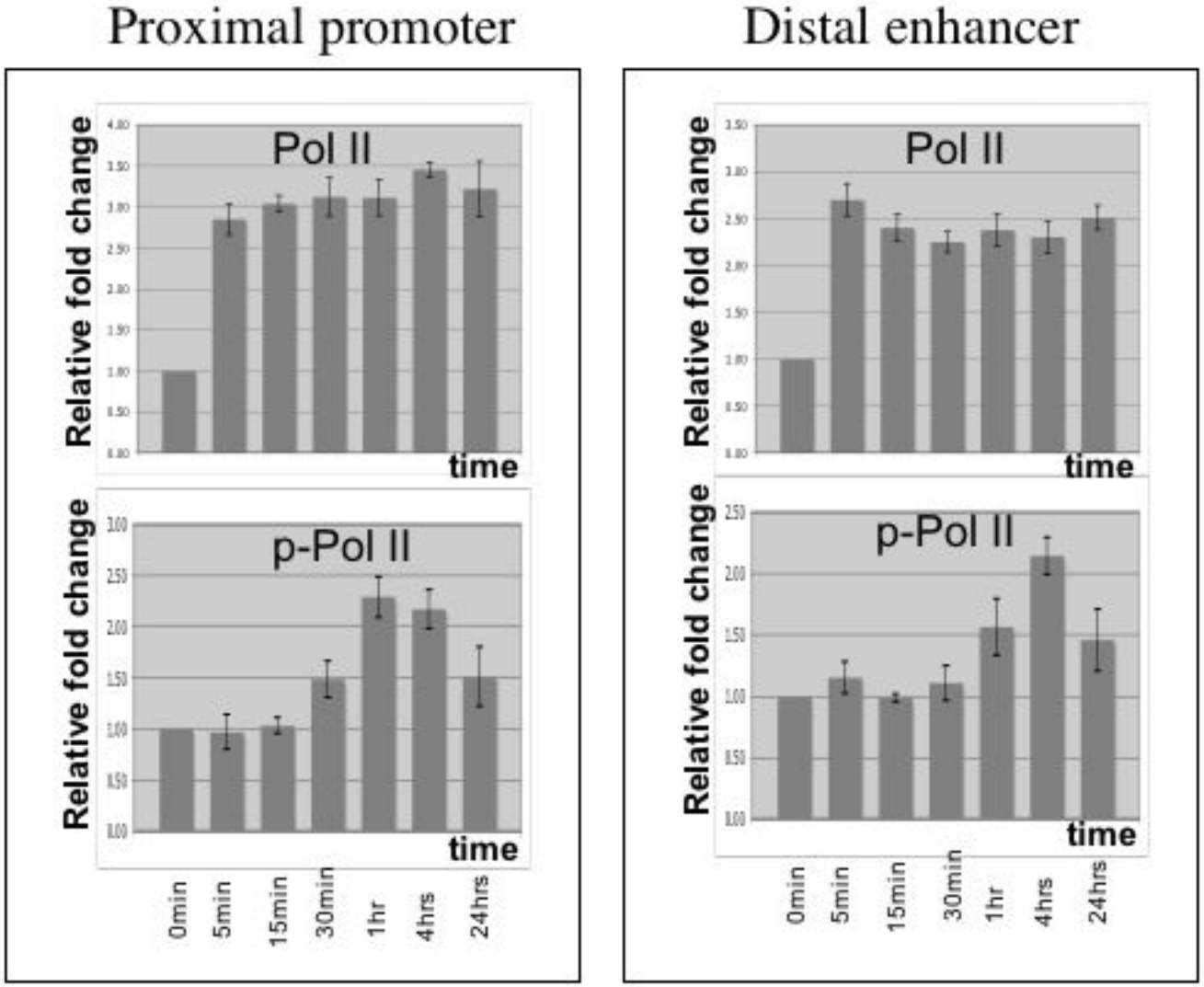

Proximal promoter
Distal enhancer
Pol II
p-Pol II
Relative fold change
time
0min
5min
15min
30min
1hr
4hrs
24hrs

Figure 11